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OPTICAL AND CHEMICAL STUDIES ON THE GRANULES IN MICROSPORES OF TRADESCANTIA

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Introduction

In certain stages in the development of the male gametophyte of *Tradescantia,* granules approximately 1–3 μ in diameter are present in relatively large numbers (pl. 31, fig. 1). They were observed by Hofmeister (1848), and Baranetzky (1880) figured them in meiotic stages in four different species. Sax and Edmonds ('33) noted that they disappear during growth and that they are relatively solid rather than fluid or plastic in consistency since they are not readily deformed by pressure on the cover-glass, and do not coalesce on contact. The latter also found that heating causes the granules to disappear.

Except for the statement of Sax and Edmonds ('33) that the granules are not composed of starch (based on a negative iodine test and a failure to observe birefringence), no information is available as to their chemical constitution. Because these granules play a significant role in certain cell processes (Johnson and Peck, '37) such information is highly desirable.

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The present investigation, undertaken at the suggestion of Dr. Edgar Anderson, is designed to furnish data of this sort.

EXPERIMENTAL RESULTS

1. OPTICAL PROPERTIES

Since the optical analysis is applicable to fresh untreated cells and granules it was entered upon at the outset of the present investigation, not only in order to obtain information as to the molecular organization of the granules, but also with the hope that it might provide a criterion for their intactness in the subsequent chemical characterization.

Young microspores of *Tradescantia paludosa* were used, these being particularly suitable if separation from the tetrad has just taken place. Because of the extremely small size of the granules, the examination in polarized light requires a highly critical adjustment of conditions of illumination and compensation. We used a standard Leitz Model BM polarizing microscope and an intense source of illumination (Leitz Universal Lamp, bulb operated at five amperes). For clarity of definition of the polarization effects it is necessary to work with the polarizer and substage diaphragms stopped down to small apertures. Under these conditions each granule presents a clear-cut polarization cross between crossed Nicols, the dark arms of the cross being parallel with the planes of polarization of the polarizer and analyzer regardless of the position of the granule in the field (pl. 31, fig. 2). The latter fact was strikingly seen in fresh preparations where the granules were in continual Brownian agitation; it was demonstrated in fixed preparations by rotation of the stage.

In order to characterize the optical properties further it is necessary to determine whether the spherites are positively or negatively birefringent (for a discussion of the technique and interpretation of the polarization optical phenomena see Schmidt, '24, '34, '37). The gypsum-plate method is inapplicable in the present problem because the granules are too small and the birefringence is too weak to determine colors in the quadrants. The more sensitive Köhler rotating compensator

was therefore used, the retardation of the mica plate being $\lambda/20$. The compensator is inserted into the draw-tube slot and rotated until the field is maximally dark, the Nicol prisms being crossed. The granules then show the typical interference cross. Rotation of the compensator slightly to one side of this position causes one pair of the quadrants of the granule to become dark while the neighboring quadrants are bright. Rotation slightly to the other side causes the picture to become reversed; the quadrants which were previously dark are now light and vice versa. From the known optical characteristics of the compensator the spherites were shown to be positively birefringent.

Because of the small size of the spherites, it is difficult to deal with their optical properties quantitatively. When the microspores are immersed in water, compensation of the cross is attained by a rotation of only 4°–5° on the compensator dial; this would correspond to a retardation of the order of 3 to 4 μμ. The diameter of the granules varies between 1 and 3 μ. On the assumption that the optical effects are due to a packing of crystallites with their optic axes (and probably also their long directions) oriented radially, the birefringence of the particles composing the granules may be calculated according to the method of Bear and Schmitt ('36, equation 11). Calculated in this way the birefringence is found to be of the order of magnitude of 0.005, a value not incompatible with the results of the following chemical characterization which indicates that the granules might be primarily of protein nature.

Immersion of the cells in balsam and in media of various other refractive indices, which was done in connection with the solubility experiments described below, shows that the form factor is relatively small, the birefringence being due primarily to intrinsic birefringence, a fact which might be expected from the apparently compact structure of the granules.

The polarization crosses, clearly visible when the optical conditions are satisfactorily adjusted, served admirably in the chemical work, not only to reveal the presence of the granules in unstained preparations but also as an index of the extent of action of the various reagents used. If the reagent had no effect on the optical phenomena it could have had little action on the

molecules, the organization and orientation of which give rise to the optical phenomena.

2. CHEMICAL AND PHYSICAL PROPERTIES

A. Microchemical Tests.—

In attempting to characterize the granules chemically, we first applied a series of microchemical tests which are more or less specific for certain groups in the proteins, carbohydrates, and fats. It was clear at the outset that such a search could be of value only if a positive test were obtained. Negative tests, while suggestive, are not conclusive because the granules are so small that unless the color developed is relatively intense it might escape detection. The tests proved uniformly negative and will therefore merely be listed briefly.

Lipoids: The granules are not stained by Sudan III and other fat-soluble dyes, nor are they blackened or even colored by osmic acid.

Proteins: The following tests for proteins or for characteristic amino-acid constituents of proteins were all negative: xanthoproteic, biuret, Millon, Raspail, Adamkiewicz, aldehyde, iodine, and lead-acetate sulphur. Certain of the tests are inapplicable to the granules as such, owing to the solubility of the latter in some of the reagents necessary for the test. For example, in applying the xanthoproteic test for protein and the Molisch test with alpha naphthol for carbohydrate, the concentrated acids dissolve the granules. Therefore, though the cell contents gave positive tests it is impossible to say that the dissolved granules were responsible rather than the constituents normally present in the protoplasm.

Carbohydrates: The following tests, based chiefly on the reducing power of carbohydrates, were all negative: Fehling's solution, iodine and potassium iodide with and without H_2SO_4, cuprammonia, chlorozinciodide, Mangin's iodine-calcium-chloride, Molisch with alpha naphthol and with thymol, ammoniacal silver nitrate. These tests were applied before and after attempted hydrolysis with 0.1 M. hydrochloric acid.

B. Enzyme Reactions.—

In the digestion experiments an objection may be raised that a negative result may simply mean that the enzyme, being a large protein molecule, may not be able to penetrate the cell wall and actually come in contact with the granules. To meet this objection, granules were also expressed from the cells and exposed directly to the action of the enzyme. The results of the experiments with diastase, pepsin, and trypsin are shown in table I.

TABLE I

THE EFFECT OF AMYLOLYTIC AND PROTEOLYTIC ENZYMES ON THE POLLEN GRANULES OF *TRADESCANTIA*. TEMP.—30° C.

Enzyme preparation	Effect on granules		
	after 48 hours	after 1 week	after 2 weeks
Diastase (sat. aq. sol.)	—	—	—
Pepsin (1% sol. in 0.1 N HCl)	—	—	—
Trypsin (1% aq. sol. pH 7.4)	+	+	+
Trypsin (1% aq. sol. pH 7.4, heat inactivated)	—	—	—

The symbol — means that the granules remain unaffected in shape and in appearance in the polarizing microscope; + means that the granules have been completely digested.

The tryptic digestion of the granules is relatively rapid and complete. Whether the negative results with pepsin mean that partial proteolysis is possible without interference with the state of aggregation of the granules, or that the material consists of relatively small molecules, is not clear.

C. Solubility Properties.—

To test the solubility of the granules in reagents the microspores were immersed in the solvents on slides and coverglasses waxed to the slides to prevent evaporation of the solvent. The slides were examined from time to time with the polarizing microscope, and the appearance and optical proper-

ties of the granules noted. Between the readings the slides were kept at a temperature of 25° C.

Organic Solvents: The granules were unaffected both in appearance and in optical properties by exposure for as long as two weeks to any of the solvents tried; these included ethyl alcohol, n-butyl alcohol, benzene, chloroform, ether, acetone, carbon bisulphide, and xylene. Similar results were obtained with mixtures in various proportions of benzene-alcohol, ether-alcohol, and xylene-alcohol, at various temperatures (see table II). This clearly rules out the possibility that lipoids enter into the structure of the granules to any significant extent.

Acids and Alkalies: Of importance in determining the general chemical nature of the granules is the action of acids and alkalies. It was found that while concentrated mineral acids readily dissolve them, they are not visibly affected by dilute mineral acids (0.1 M. HCl, HNO_3, $HC_2H_3O_2$, and H_2SO_4). On the other hand, 0.1 M. alkali (NaOH, NH_4OH) dissolves them almost completely in ten minutes and completely in thirty minutes. KOH seems to be effective only in slightly higher concentrations (0.5 M.). The process of dissolution and destruction of birefringence of the granules was readily observable under the polarizing microscope; there is no possibility that the granules were simply released from the cell by disruption of the cell membrane. On the assumption that the granules are primarily protein in nature, this effect is easily understandable, for alkali is well known to promote solution of certain proteins and by hydrolysis to cause certain relatively insoluble proteins to become soluble. Alkali is also known to have a destructive action on the birefringence of protein micelles and aggregates (Muralt and Edsall, '30). The fact that most mono- and disaccharides are soluble in dilute acid solutions, whereas the granules are not, is evidence against their being of a simple carbohydrate nature.

Urea: The granules do not dissolve even after long standing in water or dilute salt solutions (0.5 M. NaCl). However, in strong urea solutions they disappear with great rapidity. In a

typical experiment the cells were first examined in water and the presence of the granules with polarization crosses demonstrated. The urea solution was then applied. Almost before the cover glass could be adjusted and the microscope focused upon the cells, the granules had disappeared. This is not due to an optical effect, the similarity of the refractive index of the granules and the urea solution making them invisible, for in other media of similar refractive index the granules with polarization crosses were plainly visible. Moreover, the cells were subsequently stained with acetocarmine which, by staining the cytoplasm, brings out the non-staining granules more clearly; in none of the urea-treated cells could granules be demonstrated. Half-saturated solutions cause disappearance in ten minutes; lower concentrations are relatively ineffective. So long as the granules are visible in urea solutions they show a positive polarization cross.

It is known that even relatively insoluble denatured proteins are soluble in urea solution (Anson and Mirsky, '31) and that the birefringence of protein micelles may be rapidly destroyed by urea (Muralt and Edsall, '30; Bear, Schmitt and Young, '37). The action of urea therefore might be considered as further indirect evidence of the possible protein nature of the granules.

D. The Effect of Heat.—

Sax and Edmonds ('33) noted that the granules disappear on heating (no details of the temperature were given). Because of the possible bearing of the thermal data on the chemical identification, the effect of heat on the properties of the granules in a variety of media was studied. The microspores were placed on slides in the appropriate media, cover-glasses waxed on, and the slides put in a thermostated chamber, the temperature being held constant to ±1° C. After variable periods they were examined both with the ordinary and with the polarizing microscope. The data are summarized in table II. Each symbol in the table represents the effect of treatment for 24 hours at the given temperature, twenty separate determinations being made for each temperature and each medium. Ordinarily the

granules go into solution between 50° and 55° C., although there is some variability in the results and in a few instances the granules did not dissolve until boiled. No attempt was made to determine the destruction temperature with any great accuracy.

TABLE II

THE EFFECT OF TEMPERATURE ON THE GRANULES IN THE MICROSPORES OF *TRADESCANTIA*

Immersion medium	Temperature					
	35°	40°	45°	50°	55°	60°
Water	–	–	+	+	+	+
Formalin (10%)	–	–	–	–	+	+
0.1 M. HNO_3	–	–	–	–*	+	+
Alcohol (95%)	–	–	–	–	+	+
Benzene	–	–	–	+‡	+	+
Xylene	–	–	–	–	+	+

The symbol – means that the granules remain unaffected; + means that they have disappeared. There was some variation in the results, but in only two cases was this greater than 20%. * denotes a 35% exception and ‡ 40%. In the case of solvents immiscible with water the microspores were first dehydrated with absolute alcohol.

In water the granules become gradually more transparent and lose their spherical shape between 45° and 50° C. At somewhat higher temperatures they disappear completely. At temperatures slightly below their destruction temperature the bodies showed slight birefringence but no polarization crosses, possibly indicating that the original symmetry of packing of the ultraparticles had been destroyed.

Perhaps the reason why the granules of these microspores have received so little attention among cytologists is their apparent non-stainability. None of the stains used by us (safranin, fast green, methyl blue, acetocarmine, etc.) were effective, except to increase the contrast by staining the cytoplasm, the unstained granules then appearing clearly.

Another reason for the failure of the granules to appear in histological preparations is their sensitivity to heat. Except

under certain conditions, the temperature of the imbedding oven is sufficiently high to insure their destruction. It is known that certain cytological fixatives considerably increase the temperature necessary to produce shrinkage and disorganization of the protein components of certain tissues (Schmitt and Wade, '35, p. 173). To test whether fixatives tend to protect the granules against heat, anthers were placed in the following for two days: 10 per cent formalin, alcohol, and formol-acetic-alcohol. At the end of this period they were placed in the imbedding oven at 55° C. for two days. They were then removed, washed free of the fixative and the microspores examined for granules. In each case the granules were present in approximately unchanged shape. On the other hand, such material, when imbedded in paraffin and sectioned, showed no granules. The explanation of this is not clear but the best method for histological study appears to be fixation, followed by staining, running up through the alcohols and xylol, and mounting in balsam.

Summary

1. Studies have been made of the chemical and optical properties of the granules found in the microspores of *Tradescantia* in order to furnish a basis for an analysis of the role of these granules in cell function.

2. With critically adjusted optical conditions, viewed between crossed Nicol prisms, the granules present a positive spherite cross. This phenomenon has been tentatively interpreted as indicating that the granules are composed of fairly birefringent micelles oriented with optic axes radially disposed. This optical property serves admirably, not only as a criterion for the presence of the granules in unstained preparations, but for the intactness of their ultra-structure as well.

3. Microchemical tests for lipoids, carbohydrates, or proteins were inconclusive.

4. Trypsin effectively digests the granules, but pepsin and diastase are without effect.

5. Solubility data further limit the possibilities which need be considered with regard to their chemical composition. Pro-

longed extraction with a variety of lipoid solvents and of mixtures of solvents has no effect on the appearance and birefringence of the granules. They are insoluble in water, salt solutions, and dilute mineral acids but soluble in dilute alkali. The granules disappear rapidly in strong urea solutions.

6. Heating in water causes the granules to disappear in the approximate temperature range of 45°–50° C. Certain reagents, particularly certain cytological fixatives, increase the destruction temperature considerably. The possible significance of this for the histological investigation of the structures is pointed out.

7. The results so far obtained are best understood on the supposition that the granules are composed primarily of protein.

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EXPLANATION OF PLATE

PLATE 31

Fig. 1. Photomicrograph of the microspores of *Tradescantia paludosa* in ordinary light, showing some of the refractive granules. Acetocarmine smear preparation. × approx. 725.

Fig. 2. Photomicrograph of a microspore of *Tradescantia paludosa* between crossed Nicol prisms. Note that each granule presents a clear-cut polarization cross, the dark arms of the cross being parallel with the planes of polarization of the polarizer and analyzer. Acetocarmine smear preparation. × approx. 910.

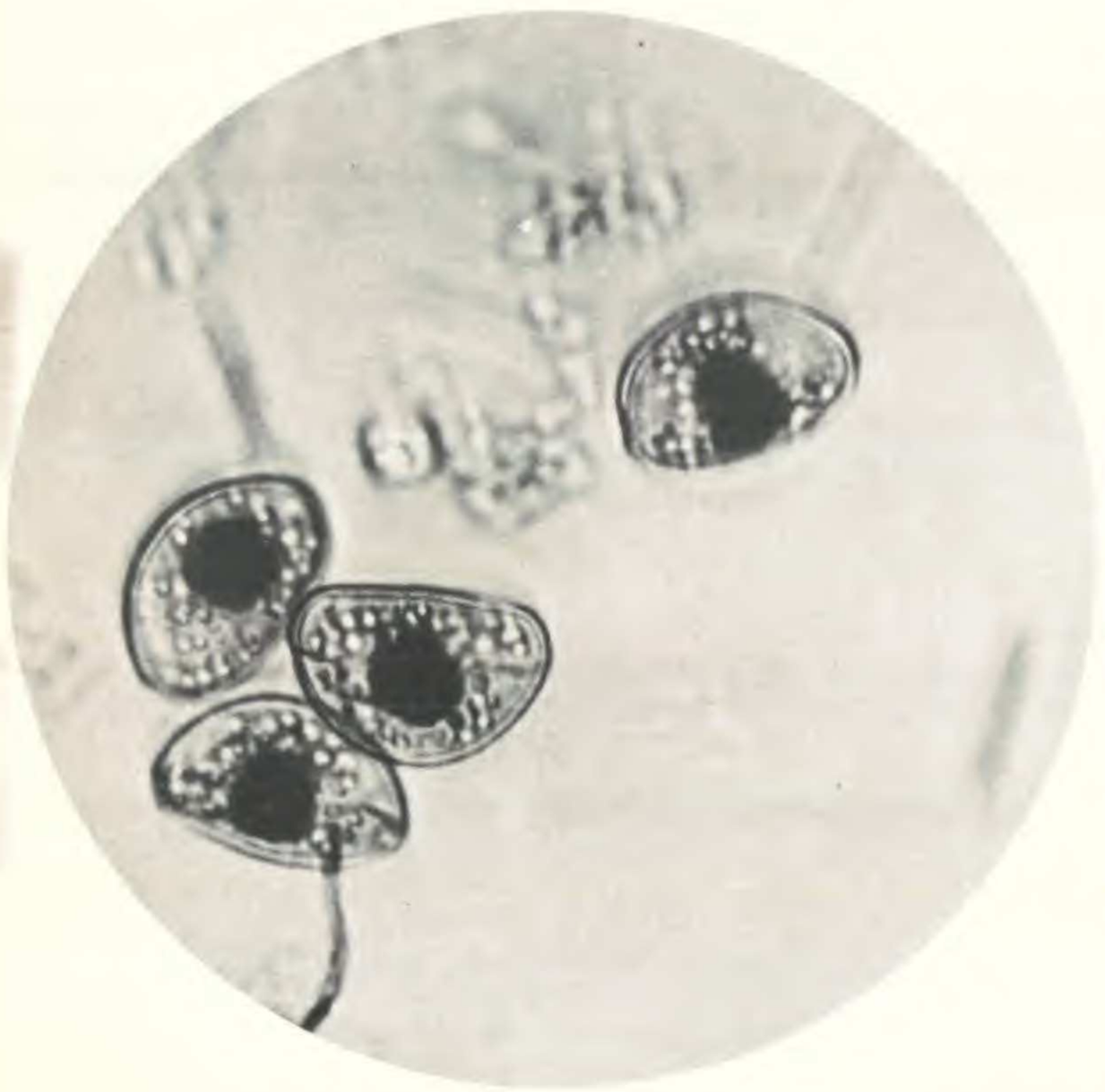

Fig. 1

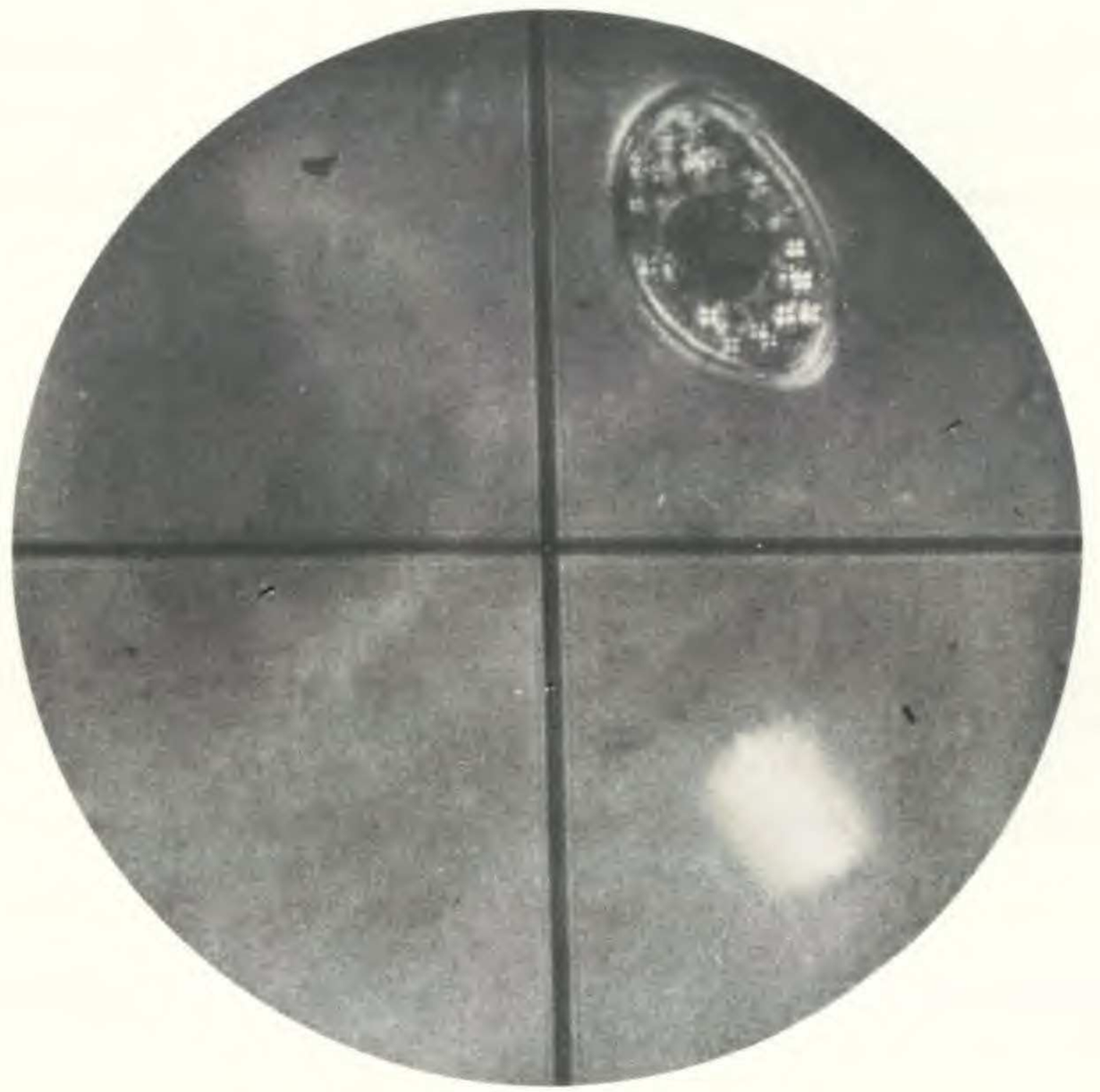

Fig. 2

SCHMITT AND JOHNSON — MICROSPORE GRANULES